Evidence for potential underestimation of clinical folate deficiency in resource-limited countries using blood tests

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ABSTRACT

Although a low serum folate concentration is a useful biomarker of pure folate deficiency, the coexistence of either vitamin-B_{12} deficiency and/or hemolysis predictably raises serum folates. Therefore in resource-limited settings where dietary folate deficiency can coexist with vitamin-B_{12} deficiency and/or malaria, the serum folate concentration can be normal-to-high, leading to serious underestimation of *tissue*-folate status. This review traces the genesis of an inappropriate overreliance on results of the serum folate concentration to rule out folate deficiency in such vulnerable populations of women and children; of significance, without due consideration of a chronically inadequate dietary folate intake, these populations have likely been wrongly judged to have an adequate folate status in influential studies. Through repetition, this error has led to a dangerous entry into the contemporary medical literature that folate deficiency is rare in these populations! As a consequence of this apologue, many millions of such women and children with mild-to-moderate *tissue* folate deficiency may have been deprived of folate replacement. This review uses historical documents to challenge earlier conclusions and re-emphasizes the need for contextual integration of clinical information in resource-limited settings.

Introduction

Food-fortification with folic acid has markedly reduced the incidence of folate deficiency and neural-tube defects wherever flour is centrally processed and equitably distributed to the general population.[1,2] However, despite such initiatives in several developed and developing countries, for a variety of reasons[2,3] up to 90% of the world's women who primarily reside in resource-limited countries are apparently still not receiving sufficient dietary folate.[4] Therefore if these women continue to consume insufficient folate during pregnancy, their progeny will begin life

with a low store of folates, which tends to propagate the problem of intergenerational folate deficiency *ad infinitum*. Yet, the contemporary literature is rife with reports from resource-limited countries that folate deficiency is less common than previously thought, and is rare, even among vulnerable populations that chronically consume a low-folate diet. What are the sources behind this bold conclusion?

There are well known limitations in use of the serum folate concentration as a biomarker for diagnosis of folate deficiency among subjects with vitamin-B_{12} deficiency and/or hemolytic diseases like malaria.[1,5,6] Thus, an intracellular metabolic block in folate utilization during vitamin-B_{12} deficiency results in raised serum folates which return to baseline only after vitamin-B_{12} replacement; therefore the finding of a normal serum folate level in such patients with untreated vitamin-B_{12} deficiency can underestimate the extent of tissue folate deficiency. Similarly, hemolysis of red cells that accompanies malaria also releases the extant 30-fold higher erythrocyte folate concentration into plasma, thereby raising the serum folate level; this too can mask true folate deficiency.

A central tenet of this review is that without sufficient consideration of the limitations of the serum folate concentration in the presence of coexistent vitamin-B_{12} deficiency and/or malaria in resource-limited countries, several investigators have categorically interpreted the results of a normal serum folate level as signifying a normal folate status. As a consequence, they have erroneously eliminated folate deficiency from consideration particularly among vulnerable populations of women and children who reside in these regions where a chronically poor folate intake is the norm. This potentially serious error from a few influential publications has led to a 'snowball effect' where continued repetition [of this error] by these and other investigators (from many disciplines of medicine) has led to the widespread conclusion that folate deficiency is rare in those very populations that urgently need folate supplementation.

Since it is so important not to miss the diagnosis of folate deficiency particularly among women and children[1,6,7]—and analysis of the literature will show this has likely been the case—the rationale for this review is to identify the genesis of how this error entered the literature, and then point to evidence from alternate sources that provides a sufficiently robust challenge to reverse this misconception.

The approach of this narrative review will be: *(i)* To provide a historical perspective on data from seminal papers which identified how associated conditions like vitamin-B_{12} deficiency and/or hemolysis predictably gave abnormal test results for serum folates [or erythrocyte folates]; *(ii)* to identify those high-profile papers from resource-limited countries that have likely misinterpreted the results of normal serum folate levels in the presence of these confounding variables (vitamin-B_{12} deficiency and/or malaria), leading to an underestimation of folate deficiency; and *(iii)* to note how amplification of this error, through repetitive citation, has become embedded as 'a fact' in the current medical literature. *(iv)* Where there is a paucity of information in the literature, an attempt has been made to frame the key questions that need to be resolved. Finally, *(v)* where interpretation of non-invasive and invasive tests remains problematic or ambiguous, the imperative for prophylaxis is discussed within the context of the geographic, socio-economic, and dietary history of vulnerable populations.

Search strategy and literature selection criteria. Data for this review was obtained by searching PubMed for all available articles published in English (up to August 2015), which contained any two of the following search terms "folate deficiency", "vitamin-B_{12} deficiency", "pregnancy", "malaria" or "hemolysis". The choice of articles that were subsequently reviewed in more detail was then based on four additional [inclusion] criteria: First, whether these articles were among the high-profile papers that originated the premise that folate deficiency was rare in resource-limited countries based primarily on the test results of serum folate levels. Second, the

studies were conducted in populations where there was no consumption of folate-fortified food or multivitamins. Third, whether there were source documents from other contemporary studies or other disciplines that provided insight into the prevailing nutritional or social context of these populations, and if such documents pointed to a high likelihood of coexisting nutritional vitamin-B_{12} deficiency and/or malaria. Fourth, if such revelations allowed for a sufficiently strong challenge to seriously question the veracity of the earlier conclusions.

Shortcomings in the diagnosis of tissue vitamin deficiency when blood tests for vitamin-B_{12} and folate are solely used — *the importance of clinical details*. In several papers analyzed in this review, studies on the vitamin-B_{12} and folate status of subjects in resource-limited countries were conducted in the absence of clinical details. Such studies are incomplete because they fail to provide information on dietary intake or other clinical symptoms and/or signs, and on whether there is associated megaloblastic anemia (reflective of tissue deficiency of either vitamin-B_{12} or folate) and/or neuropsychiatric abnormalities (reflective of tissue deficiency of vitamin-B_{12}). Such information is important because it is well established that even when subjects with vitamin-B_{12} deficiency are ambulatory, megaloblastic anemia can be present without major symptoms. For example, it is not uncommon for subjects in India and African countries to be fully ambulatory with megaloblastic anemia and hemoglobin levels of 9-10 grams/deciliter (or lower) with minimal to no symptoms; this is because the body has compensated over the long duration it takes to develop vitamin-B_{12} deficiency.[1,6,8] Such symptoms can often be only elicited by leading questions in conjunction with a comprehensive physical examination[1,9]. Moreover, among ambulatory subjects with biomarkers that suggest vitamin-B_{12} deficiency, it may even require sophisticated neurological testing to reveal evidence for subtle abnormalities that reflect tissue deficiency of vitamin-B_{12}.[10-12] It should be noted that in resource-limited countries, apart from a primary dietary deficiency of folates, when vitamin-B_{12} deficiency is prolonged,

megaloblastosis of the intestinal cells can also predispose to a secondary malabsorption of [the already meager intake of] dietary folate; this can lead to folate deficiency developing late in the course of vitamin-B_{12} deficiency.[8,13] Therefore in the absence of clinical data, the extent of tissue deficiency of vitamin B_{12} deficiency and/or folate cannot always be definitively ascertained merely from the serum level.[1,6,8]

Limitation of the serum folate biomarker in the setting of vitamin-B_{12} deficiency — *a historical perspective.* In the early days, microbiological assays using bacteria that thrived on folate for their growth were used to measure the serum and erythrocyte folate concentration. Using such assays, hematologists in North America and the United Kingdom established that patients with anemia due to folate deficiency had low serum and erythrocyte folate concentrations (***Table*** 1); this made it a useful standardized and objective measure in the clinical assessment of folate status in patients with anemia. However, when these investigators searched for other clinical conditions that could consistently raise the serum folate concentration, they found two confounding variables: *i)* patients with vitamin-B_{12} deficiency, and *ii)* hemolysis of red blood cells. Thus, as early as 1962, among 100-consecutive patients with pernicious anemia, megaloblastic anemia, and a low serum vitamin-B_{12} less than 100-pg/ml, Herbert and Zalusky identified that "one-third with vitamin-B_{12} deficiency had falsely high serum folate levels".[14] These pioneers were remarkably prescient in their suggestion that in the presence of mild-to-moderate tissue folate deficiency, the serum folate would be in the *normal* range in the presence of vitamin-B_{12} deficiency. Likewise Waters and Mollin[15] observed in 1963: **"**The highest serum folate (L. casei) levels were found in patients with the lowest serum vitamin-B_{12} levels. As the serum vitamin-B_{12} level increased there was a progressive fall in the serum folate level…" In 11-patients, they observed "[a] rapid fall in the mean serum folate level to approximately a third (34%) of the pre-injection mean during the first 24 hours after an injection of 1000-µg of

vitamin-B_{12}".[15] Thus, if the serum folate concentration was at a low-normal range at baseline (prior to vitamin-B_{12} injection), the subsequent reduction in serum folate levels after vitamin-B_{12} could easily have dropped into a folate-deficient range.

Indeed, a year later, Cooper and Lowenstein[16] documented a predictable movement of previously high serum folate values towards lower [baseline] levels in all 10-patients with pernicious anemia following correction of vitamin-B_{12} deficiency. Similarly, after vitamin-B_{12} replacement of 15-Indian children (aged 6-months to 12-years) who were suffering from nutritional anemia due to vitamin-B_{12} deficiency,[17] the serum folate concentration dropped in 6 of 15-patients (40%) from previously normal values (mean of 17·6-ng/ml) to a deficient level with a mean of 4·8-ng/ml (their cut-off values for folate deficiency were less than 6-ng/ml)[18]; this suggested that this cohort likely had *associated masked* folate deficiency. In addition, among 5 of 15-patients, serum folates remained persistently low even after vitamin-B_{12}, consistent with *associated overt* severe folate deficiency. Thus, whereas 11 of 15-patients (73%) of these children actually had a <u>combined</u> vitamin-B_{12} *plus* folate deficiency, *a full 40% of patients with folate deficiency would have been missed if only a single serum folate test value was available*. [This is an important point because we will shortly see how other clinical investigators in resource-limited countries have erroneously made the diagnosis of 'pure' vitamin-B_{12} deficiency from only a single blood test result (that revealed a low serum vitamin-B_{12} but normal folate levels), which has likely missed significant numbers of patients with associated folate deficiency (***Table*** 1)].

Nixon and Bertino[19] then determined that accumulation of serum folate in patients with vitamin-B_{12} deficiency (pernicious anemia)—and its fall after treatment with vitamin-B_{12}—involved decreased clearance, reduced cellular metabolism, and increased renal excretion of 5-methyl-tetrahydrofolate. These results were consistent with the "methyl-folate trap" hypothesis—reflecting

poor utilization of folate during vitamin-B_{12} deficiency to explain why hematological manifestations of vitamin-B_{12} deficiency resembled folate deficiency (***Figure*** 1). Parenthetically, Nixon and Bertino[19] also predicted that administering folate to vitamin-B_{12}-deficient subjects would lead to "a several-fold elevation of the total serum folate concentration…" Indeed, fifteen years later, among a cohort with vitamin-B_{12} deficiency, Stabler *et al*[20] documented that those consuming folate-rich multivitamins had the highest recorded serum folate values.

Is there a threshold whereby there is a failure in maintaining the inverse relationship between low serum vitamin-B_{12} and higher serum folate? Thus far, we have considered the close relationship between a low serum vitamin-B_{12} level and an inverse rise in serum folate level among subjects with clinical findings of anemia attributable to vitamin-B_{12} deficiency. Whether such an inverse relationship holds throughout the spectrum of severe-, moderate-, and mild- vitamin-B_{12} deficiency, or there is a particular threshold level (such as in mild serum vitamin-B_{12} deficiency when megaloblastic anemia has not yet manifested clinically) where this inter-relationship is not sustained, is not known. However, it is well established that the metabolic effects of vitamin-B_{12} deficiency that lead to a functional (and later real) intracellular functional folate deficiency[21] *always precede* the morphological manifestations of megaloblastic anemia.[1,6,8] Therefore it would appear that this inverse relationship in serum vitamin-B_{12} and folate levels would hold firm even in mild-to-moderate vitamin-B_{12} deficiency, and occur even before clinical findings of nutritional anemia were manifest. This is consistent with the earlier clinical observations[15] and the dynamic changes in serum folate upon replenishment of vitamin-B_{12}.[17] This is an area for future investigation.

Red blood cell (RBC) folates as a measure of folate stores — *a historical perspective*. Unlike the serum folate, which is entirely 5-methyl-tetrahydrofolate, RBC folates consist of a heterogeneous mixture of different coenzyme forms with varying polyglutamate chain lengths. Clinical measurement of RBC folates took on importance when tissue folate deficiency was clinically

correlated with a decrease in RBC folate (less than 150 ng/ml) by a microbiological assay in the 60's[22]. Thus finding a decreased serum and RBC folates with megaloblastic anemia was consistent with the diagnosis of folate deficiency. Although this seemed like the ideal combination to diagnose folate deficiency, RBC folate tests had major limitations in sensitivity and specificity[8,23,24], and were notoriously unreliable in alcoholics and in pregnancy; thus, it was normal/borderline in approximately 30% of alcoholics[23] and 60% of pregnant patients[25] with clinically significant folate-deficient megaloblastic anemia. Furthermore, a reduction of RBC folate also occurs in approximately 60% with vitamin-B_{12} deficiency, further limiting its diagnostic utility.[8]

There is a further twist to this issue: the original microbiological assays for RBC folates have been replaced by radioassays currently used for measurement of *serum* folate levels. Apart from a lack of clinical validation of the use of *radio*assays for measuring RBC folates, these radioassays have had a long history of being unreliable with respect to lack of precision and accuracy, generally leading to a lack of confidence in the results.[26] In addition, a quality assurance study from Australasia involving 134 laboratories identified nine (operator controllable) factors which could also lead to poor RBC folate results.[27] And later analysis of its clinical value led to the conclusion that the RBC folate test added little useful information to that provided by serum folate levels.[28] Together, these clinically relevant issues have considerably dampened enthusiasm for use of RBC folate tests, leading several experts to entirely avoid their use pending a resolution of problems related to reagent batch quality control, quality assurance, and equally important, provision of data on clinical validation by the various manufacturers of these kits.

Therefore because of intrinsic problems of poor validation and unreliability, commercial RBC folate assays are not routinely used in the clinical diagnosis of folate deficiency[1,29]; so now, serum folates are primarily used for population-wide assessment of folate status in resource-rich

settings[30-32] as well as for routine clinical diagnosis of folate deficiency.[1,6] However, as noted above, the serum folate test has limitations in resource-limited populations where consumption of both vitamin-B_{12} and folate are poor.

Clinical use of sensitive metabolite levels to diagnose vitamin-B_{12} and/or folate deficiency — *a historical perspective.* By the late 1980's, the combination of serum homocysteine and serum methylmalonic acid tests were introduced clinically as far more sensitive diagnostic markers of vitamin-B_{12} deficiency than measurement of serum vitamin-B_{12} concentrations.[20,33] However, it should be noted that the serum homocysteine is also increased in folate deficiency[6,20,33] (***Table*** 2). Therefore although high serum homocysteine and methylmalonic acid levels can reflect underlying vitamin-B_{12} deficiency, a combined vitamin-B_{12} *plus* folate deficiency cannot possibly be ruled out by a single set of tests[6,20,33]; see ***Table*** 1 and ***Table*** 2. Indeed, the only way to prove that a patient has *pure* vitamin-B_{12} deficiency involves a laborious, costly, and impractical approach of serial *reassessment* of both the serum homocysteine and methylmalonic acid values *after* vitamin-B_{12} replacement. Thus, with pure vitamin-B_{12} deficiency, both serum homocysteine and methylmalonic acid should drop completely into the normal range; however, if folate deficiency was also present, although the serum methylmalonic acid concentration would normalize, the serum homocysteine would not completely drop into the normal range.[34] Needless to state, except for isolated exemplars[35], such detailed studies using metabolites[20,33] have not been reported among large cohorts of subjects from resource-limited settings. But by the same token, any study purporting to having diagnosed patients with 'pure' vitamin-B_{12} deficiency—(using evidence from a single blood test showing low serum vitamin-B_{12} and normal serum folate values, and/or combined elevated serum methylmalonic acid and serum homocysteine values)—cannot have unambiguously ruled out an associated folate deficiency, particularly in resource-limited settings where dietary folate intake is persistently inadequate.

Yet, many high-profile studies have made precisely such claims, as discussed below.

Masking of folate deficiency with coexistent vitamin-B_{12} deficiency

1. Non-pregnant adults in India. When Refsum *et al*[36] evaluated an ambulatory population in Pune with a single set of blood tests (without dietary *or* clinical information), they reported that 47% had serum vitamin-B_{12} values that were consistent with vitamin-B_{12} deficiency (less than 150-pmol/L), but apparently less than 5% had evidence of folate deficiency based on serum folate levels (less than 5-nmol/L). In addition 77% had hyperhomocysteinemia (more than 15-μmol/L) and 73% had elevated serum methylmalonic acid (more than 0·26-μmol/L). On this basis, these investigators correctly concluded that vitamin-B_{12} deficiency was widespread among three-quarters of the population studied. However, their conclusion *that folate deficiency was not a major problem* is incorrect[36] because only a single blood sample was assessed, and intentional studies designed to specifically unmask associated folate deficiency—by longitudinal measurement of serum folate values before and after vitamin-B_{12} replacement[16,17,34]—were not conducted. Because homocysteine is elevated in both vitamin-B_{12} and folate deficiency, these investigators did not unambiguously rule out an associated folate deficiency; this could have been shown out by first demonstrating a failure to drop the serum homocysteine into the normal range after vitamin-B_{12} therapy, and then confirming a further drop in serum homocysteine into the normal range only upon addition of folate replacement]. Therefore, Refsum *et al*[36] have not unambiguously ruled out a combined vitamin-B_{12} *plus* folate deficiency.[37] Because the *average* serum folate was 12·2-nmol/L [or 5.38-ng/ml] (i.e., in the low-normal range of the assay which had an upper limit of approximately 45-nmol/L)—and based on the potential for masking of folate deficiency during vitamin-B_{12} deficiency[15-19,22]—it is quite possible that vitamin-B_{12}-replenishment could have led to either a drop of serum folate concentrations below the lower limit of normal, or an incomplete drop of homocysteine into the normal range. Indeed, a

subsequent study from this region provided precisely such evidence to support the possibility of an associated folate deficiency[38]: Thus, in a small subgroup of non-pregnant women from Pune, following replacement with alternative days of 500-μg oral methyl-vitamin-B_{12} over 6 weeks,[38] these investigators observed: "Normalization of tHcy [total serum homocysteine] was obtained in only 4/11 women, while seven women continued to be hyperhomocysteinemic despite improvement of vitamin-B_{12} status within 'normal' range and despite a normal folate status." However, the serum folate value of those women believed to have "a normal folate status" was actually in the borderline *low-normal range*, with a median value of 4.4-ng/ml at baseline (25%, 75th centile = 2.4- and 5.8-ng/ml, respectively, with a cut-off value of less than 3.0-ng/ml signifying frank folate deficiency).[38] Therefore women at the '25th centile' clearly had laboratory evidence of folate deficiency, whereas many others had values in the low-normal range; and because they were not replenished with folate, their serum homocysteine likely failed to drop into the normal range, as would be predicted in a population with combined low vitamin-B_{12} and folate status. Equally revealing was the finding that "green leafy vegetables did not alter the serum folate or tHcys [total serum homocysteine]".[38] This reflects the well-known low bioavailability of folate in such foods after cooking.[1,39] The existence of nutritional folate deficiency among these women should not be surprising because this predominantly vegetarian and near-vegetarian[7,37] population subsists on well-cooked lentils, beans, and vegetables and only small portions of dairy (yogurt/milk), occasional eggs and seasonal fruit; moreover fresh salad greens are not customarily consumed. Even the non-vegetarian population consumes a similar diet as vegetarians but with the inclusion of only small portions of curried meats—at most twice a week.[7,37] Therefore, in analogy to the data from Saraya *et al*[17] in Indian children, there were a number of adult women in the study by Yajnik *et al*[38] who were already folate-deficient; this fact could *prevent* a rise from preexisting low levels (following vitamin-B_{12} replacement) into the

normal folate range as had been previously observed in Indian children.[17] While one cannot state with certainty—because intentional longitudinal replenishment studies have not been carried out—when taken together, these issues support the likelihood that the population studied in Pune[36] had many more patients with combined vitamin-B_{12} *and* folate deficiency[37] than the publication originally suggested. Yet the repetitive misinterpretation of findings of a single normal serum folate level as ruling out folate deficiency in this population[36] has evolved over the years into a much more definitive assertion—as exemplified in this abstract[40]: "Two thirds of the mothers had low vitamin B_{12} concentrations, *folate deficiency was rare,* and high circulating concentrations of homocysteine predicted IUGR" [*Italics mine*]. It cannot be overemphasized that such a conclusion from this dedicated nutrition group in India is both erroneous and misleading to less specialized healthcare givers at large. [Parenthetically, prospective longitudinal follow up after individual serial vitamin-replacement can unambiguously answer the question of the frequency with which the serum folate can fail to rise into the normal range in such populations with combined vitamin-B_{12} and folate deficiency].

2. Pregnant women in India. Folate deficiency during pregnancy arises from a combination of poor dietary folate intake, increased requirements for maternal-fetal growth, and increased losses.[1,6] The majority (more than 90%) of women of reproductive age in resource-limited countries (from sub-Saharan Africa, South and South East Asia, and Latin America) consume less than the estimated average requirement of folate,[4] which predisposes them to folate deficiency before, during, and after pregnancy. By the late 1960s using microbiological blood tests for serum folate, folate deficiency was diagnosed among 85-100% of Nigerian,[41] up to 80% of Canadian[42] and 73% South Indian women[43] in the third trimester of pregnancy. Parenthetically, if these South Indian women also had metabolic evidence of vitamin-B_{12} deficiency—which we now know to be a worldwide problem[7,33,36,37,44]—the finding of a below-

normal serum folate concentration in these women would actually reflect *severe* tissue folate deficiency.

But do South Indian women consume less folate then their sisters in North India? The estimated dietary intake of folate among women in Northeastern and Eastern States in India is only 75- to 168-μg/day[45]; these values range from one-fifth to one-third of the optimum needed to prevent neural-tube defects.[1,6] Despite these facts, among 283-pregnant women (one-half who were less than 22-years old) from rural Haryana in North India, 68% had iron deficiency, 74% had vitamin-B_{12} deficiency, but only 26% apparently had folate deficiency based on blood tests.[46] The implied conclusion was that vitamin-B_{12} deficiency is more of a threat than folate deficiency.[46] But since vitamin-B_{12} deficiency was also present, many of those women with mild-to-moderate *tissue* folate deficiency could be expected to have serum folate concentrations in the 'normal' range.[17] An earlier study from this region[47] noted that "the consumption of food groups rich in micronutrients (pulses, vegetables, fruits, nuts and oil seeds, animal foods) was infrequent"; that "99% of these women consumed less than one-half of the recommended folic acid"[46]; that "over 19% pregnant women were consuming less than 50% of the recommended calories",[47] and 60% of pregnant women consumed less than 75% of recommended daily caloric allowance "indicating an overall poor food intake".[46] Such levels of poverty strongly correlate with and support the likelihood of low-folate status.[1,6] Moreover, the state of Haryana is adjacent to Delhi—where both affluent city-folk and neighboring slum-dwellers were also documented to have equally poor dietary intake of folate *and* vitamin-B_{12}, with high homocysteine values of 23- to 25-μM among 88% of study subjects.[48] Again, such results were likely due to a combination of folate plus vitamin-B_{12} deficiency because more than 90% of <u>both</u> groups consumed less than one-fifth of optimum folate, and more than 75% consumed less-than-optimum amounts of vitamin-B_{12} daily.[48] Therefore it is likely that many of those pregnant women from rural Haryana

[46] had a combined vitamin-B_{12} *plus* folate deficiency (with the latter masked by extant vitamin-B_{12} deficiency). So it should be stressed that folate deficiency is pervasive among women in both South and North India.

These case studies illustrate how the use of biomarkers of serum folate without due consideration of the prevailing nutritional intake of folate and vitamin-B_{12} has led to a systematic de-emphasis of the issue of masked folate deficiency (within the context of widespread vitamin-B_{12} deficiency). This can continue to perpetuate the wrong concept that folate deficiency is not a serious problem among women residing in resource-limited countries. Moreover, any consequent reduction in vigilance over the need for folate supplements for *young girls well before they become pregnant* has the dangerous potential to perpetuate maternal folate malnutrition with serious consequences to maternal-child morbidity and mortality.[49]

Limitation of the serum folate biomarker in the setting of hemolysis (malaria) — *a historical perspective.* In 1961, Waters and Molin[50] observed: "[It] was found that varying degrees of hemolysis increased the folic acid activity from three to ten times that of unhaemolysed serum". By 1966, Hoffbrand *et al*[22] documented a 30-fold *higher* erythrocyte folate to serum folate ratio in normal individuals. Therefore profound hemolysis during the erythrocytic stage of *Plasmodium falciparum*, which infects erythrocytes of *all* ages *including* bone marrow orthochromatic erythroblasts,[51] will release their 30-fold higher folates into serum.[22] *In vitro* hemolysis can also occur during blood collection, transportation, or storage arising from inadequate resources and/or infrastructure in malarious areas.[52,53]

Intravascular hemolysis of parasitized cells and hypersplenism induces a powerful stimulus for compensatory erythropoiesis,[54,55] increasing basal erythrocyte-production rates (of 2-million per second in humans) by 5- to 10-fold, leading to significant reticulocytosis. However, over 50-years ago, Herbert and Zalusky[14] reported that *reticulocytes* are richer in folate than

mature erythrocytes; thus 26% reticulocyte-rich preparations had nearly 10-fold increases in folate content than 6% reticulocyte-poor preparations (i.e., 273-ng/ml *versus* 29-ng/ml, respectively). Parenthetically, the physiological basis for higher folate content of reticulocytes and erythroid precursors was determined over two decades later in my laboratory.[56-58] Both reticulocytes and mature erythrocytes contained vestigial remnants of functional folate receptors that primarily mediated the uptake of folate into erythroid precursors within bone marrow.[58] Thus, in the presence of compensatory erythrocytosis and reticulocytosis associated with hemolysis, analysis of such reticulocyte-rich blood for erythrocyte folate content (RBC folate concentration) would yield high values; this could give a false impression of normal folate status, when in fact, the true folate status may be low because of a combination of poor dietary folate intake and increased folate requirements to support compensatory hematopoiesis (***Table*** 1). (The extent of contribution to the red cell folate content from *Plasmodium falciparum,* which can synthesize folates in erythrocyte cultures *in vitro*[59] and also raise erythrocyte folates in animal models with high levels of parasitemia,[60] is unknown in humans). So measurement of the serum folate level in malaria during hemolysis of *Plasmodium falciparum*-infected erythroid precursors, reticulocytes, and mature erythrocytes, with release of folate-rich intra-erythrocyte contents (and an as yet unknown quantity of liver folate) *into* serum, would also provide misleading estimates of the patient's folate status. In this context, there would be added complexity because repeated cycles of *Plasmodium falciparum*-induced hemolysis would release substantial amount of *various forms of folate*—i.e., 5-methytetrahydrofolate [monoglutamates] and folate polyglutamates of different glutamate chain lengths[61]—*into* the serum. Apart from the poor clearance of serum 5-methyltetrahydrofolate that is associated with vitamin-B_{12} deficiency[19] (***Figure*** 1), polyglutamylated-folates are also inefficiently transported back into cells relative to monoglutamates.[1,62] However, because the earlier microbiological assays for folate could not

discriminate between these various forms of folates[22,50] a high serum folate would be reported in all these individuals (***Table*** 1).

Populations living in resource-limited malarious regions of Asia and Africa often suffer from food insecurity, which predicts for poverty-imposed near-vegetarianism[1,6,7]; this involves consumption of a monotonous, largely vegetarian or vegan-like diet that is low in folate, iron, and vitamin-B_{12}.[7,37,44,63] Here too, the coexistence of vitamin-B_{12} deficiency in these populations will also raise the serum folate concentration.

There is no prior human data that has systematically evaluated the effects of repeated cycles of *Plasmodium falciparum*-induced hemolysis on serum folate levels; however, given the longstanding mandate of administering prophylactic folate supplements for <u>*all*</u> patients with hemolysis,[1,6] it would be unethical to prospectively conduct such studies. Nevertheless, because <u>*evidence*</u>—(i.e., 30-fold higher erythrocyte folate; hemolysis of folate-rich erythrocytes, reticulocytes, and orthochromatic normoblasts during the erythrocytic phase of malaria; and associated vitamin-B_{12} deficiency)—*does compound*,[64,65] there is a high probability that in resource-limited settings associated with food insecurity-imposed <u>inadequate</u> vitamin-B_{12} *and* folate intake, a patient with profound hemolysis from *Plasmodium falciparum* malaria will <u>still</u> have an artificially high [or normal] serum folate concentration despite extant nutritional [tissue] folate deficiency.

With this background, we are in position to critically evaluate the veracity of conclusions from influential studies that measured serum folates in African children with malaria and/or vitamin-B_{12} deficiency and [erroneously] concluded that folate deficiency is uncommon, *even though their diet was severely compromised by dire poverty and/or famine*.

Masking of folate deficiency by malaria (and probable nutritional vitamin-B_{12} deficiency)

(i) Children in The Gambia. Abdalla *et al*[66,67] observed that "the serum and red cell folate levels

were *above* the lower limits of normal" among 75 of 106-Gambian children less than 5-years with acute uncomplicated *Plasmodium falciparum* malaria and variable degrees of anemia. These investigators therefore concluded that folate deficiency was not likely given these laboratory values. Unfortunately however, they failed to make connections between the potential for malaria-induced hemolysis *and* reticulocytosis to raise the serum and red cell folate concentration independently of the folate status of these children. But were these children well nourished and consuming a well-balanced diet that was rich in folates? To the contrary, historical records[68] reveal that there was a very high likelihood of severe food insecurity in The Gambia during this period. For example, Abdalla *et al* conducted their series of studies during harsh, recurrent droughts of the 1970's and 1980's (known as the [African] 'Sahel Desiccation') that included The Gambia, which resulted "…in massive losses of agricultural production and livestock; loss of human lives to hunger, malnutrition and diseases; massive displacements of people and shattered economies".[68] So there was evidence of severe food insecurity, which predisposes to poor dietary intake of micronutrients (folate *plus* vitamin-B_{12}) and iron.[7,37,44,63] Yet, without considering *a)* the high red cell folate concentrations in reticulocyte-rich blood, or *b)* the role of hemolysis of *Plasmodium falciparum* infected erythroid precursors, reticulocytes and erythrocytes in increasing the serum folate, or *c)* the likelihood of associated dietary vitamin-B_{12} deficiency in artificially raising the serum folate,[22,50,52,53] or *d)* the need to provide folate to support compensatory hematopoiesis (the prevailing dictum[8,13]), or *e)* the extant poor nutritional status of these children, these investigators (hematologists) did not recommend folate supplements. As a result, other clinicians and nutritionists have been misled by a fallible laboratory test over other indicators of folate deficiency. These influential studies[66,69-71] have led to an across-the-board conclusion that tens-of-millions of other children with chronic hemolysis from repeated episodes of malaria *and* a poor dietary intake of folate do not have folate

deficiency.[71,72] Equally surprising, is the fact that innumerable papers and authoritative reviews on anemia in malaria[73,74] have not even considered the likelihood of poor micronutrient intake as a contributing factor to anemia in these populations. As a consequence, there has been no mandate from the medical establishment for giving such children folate despite their likely need of this vitamin.

(ii) Children in Malawi. In a high-profile paper, Calis *et al*[75] found severe anemia in Malawian children where malaria was found in 60% and vitamin-B_{12} deficiency in 30% "[but] folate and iron deficiencies are not prominent". In response to the issue of extant poor diet, poor test-sensitivity, and hemolysis in underestimating folate deficiency [raised in correspondence[76]], the authors acknowledged: "[It] is theoretically possible that hemolysis and vitamin-B_{12} deficiency have masked folate deficiency…. We did not evaluate the source of dietary-folate intake in these children in detail, but green vegetables and fruits account for a large proportion of the dietary intake of children in Malawi…".[75,76]

However, this rosy dietary assessment among children in Malawi—the seventh poorest country in the world—is contradicted by reports from *three* different sources that highlight the nutritional status of the population at that time:

(a) A Food and Agriculture Organization report,[77] which highlighted two drought-induced famines in the 1990s and in the year immediately preceding and following the 2002-2004 study by Calis *et al*,[75] concluded with a poignant description of serious malnutrition among Malawian children less than 5-years—the age group studied[75]: "…[p]overty and food insecurity explain the very high prevalence of chronic malnutrition that plagues Malawi, almost half of the children under-five years being stunted… Micronutrient deficiencies are widespread…. The nutritional status of the Malawian population remains critical".[77]

(b) An earlier study on the impact of malarial infection and diet on anemia in rural pregnant Malawian women in 1999 noted[78]: "Cereals, predominantly maize, provided more than 75% of energy in their diets. Maize flour has a very low folate content (that is 25-μg/100-gm). It is consumed two to three times a day, often as a stiff porridge (*nsima*) with a cooked relish prepared from green leaves (for example pumpkin leaves) or legumes. However, because the traditional Malawian cooking practices involve protracted boiling, much of the folate in the cooked relishes is probably destroyed".

(c) The intergenerational (mother-to-child) transmission of micronutrient deficiency has a profound impact on children. Among 150-anemic Malawian women predominantly in the third trimester,[79] folate deficiency was found in only 34%, and vitamin-B_{12} deficiency in 33%—(80% of whom also had normal serum folates). However, a then-contemporary Malawian report in 2000 noted the serious food insecurity and famine-like conditions of the nineties, and concluded[80]: "Young children and women of reproductive age are especially vulnerable to nutritional deficits and micronutrient deficiency disorders". Because folate-rich foods are not easily available[78] and consumption of folate supplements during socio-economic upheavals of famine are uncommon,[1,6] it is quite unlikely that these Malawian women consumed a folate-rich diet sufficient to avoid folate deficiency during the third-trimester. So how can one explain the observed 'normal serum folate levels' among two-thirds of this pregnant population?[79] Here, it is particularly significant that these authors noted[79]: "The diet of most women in our situation is effectively vegan". Although such vegan diets are notoriously low in vitamin-B_{12} content mandating routine prophylactic vitamin-B_{12}-supplementation,[1,7,81-83] this was not routine practice in Malawi.[79] Therefore, it is likely that a greater number of these Malawian women[79] would have been diagnosed with vitamin-B_{12} deficiency if more sensitive [metabolite] studies of serum methylmalonic acid and homocysteine were used.[7,33,36,44,63] And many more women with 'normal'

serum folate in that study[79] likely had masked tissue folate deficiency. Such women would invariably pass down their own folate/vitamin-B_{12} deficiency to their babies, who continue to consume folate/vitamin-B_{12}-poor breast milk[35,84,85] before being weaned onto similar diets as their mothers.

So how did those little Malawian children in the study by Calis *et al*[75,76]—who needed folate to support compensatory hematopoiesis *and* were in the midst of near-famine conditions in Malawi[77,79]—acquire folate and avoid folate deficiency if their mothers were not getting sufficient vitamin-B_{12} or folate? The body of evidence cited[77-79] is contrary to the assertion that an abundant folate-rich diet consumed by those children can explain findings of 'normal serum folate' values in their patients.[75,76] Yet, the conclusion that folate deficiency is not a problem in patients with malaria[75] (which mimic earlier conclusions[66,69-72]) is now widely viewed as factual: The authoritative UpToDate® entry on 'Anemia in Malaria',[74] which highlighted the work of Abdalla[67] and Calis *et al*,[75] concluded that folate deficiency is uncommon[74]: "*Although dietary deficiencies are widespread in malarial endemic regions* the influence of reduced folate levels [*sic*] are *not* thought to be major contributors to the dyserythropoiesis seen during severe malarial anemia.[67] The finding of low vitamin B_{12} levels in malaria suggests that subclinical deficiency of B_{12} may play a hitherto unrecognized contribution to severe anemia…[75]". —[*Italics mine*].

(iii) Children in Kenya. The case from Embu, Kenya, is instructive because both vitamin-B_{12} deficiency and *Plasmodium falciparum* malaria were endemic in the region.[86] Among children aged 5- to 14-years, 31% had serum vitamin-B_{12} levels less than 125-pmol/L (*severe* vitamin-B_{12} deficiency) whereas 38% had serum vitamin-B_{12} values between 125-220-pmol/L (*moderate* vitamin-B_{12} deficiency). Although one-third had malaria, 45% had enlarged spleens (suggestive of chronic malaria re-infection). However, the prevalence of folate deficiency *based on serum folate levels* was almost zero! (The actual values for serum folate were between 20-46-nmol/L

with the upper value at the upper limit of the assay). Of significance, the potential role of vitamin-B_{12} deficiency or malarial-hemolysis in artificially raising serum folate (thereby masking an underlying folate deficiency) was not considered.

Taken together, despite the known limitations of the blood tests for folate in the presence of vitamin-B_{12} deficiency and/or hemolysis accompanying malaria, there has been a consistent undue overreliance on these imperfect blood tests, even over the more reliable estimates from poor dietary folate intake and other clinical indicators of folate deficiency (discussed below). Because the results of such blood tests invariably trigger decisions for [or against] replacement therapy, the interpretation of results following the measurement of the serum folate level in these patients has, in all likelihood, resulted in the therapeutic decision to [continue to] withhold folates from these unfortunate women and children, thereby predicting an adverse outcome.

Tracing the potential cognitive biases in medicine that may have led to propagation of an error in the literature. How could the medical establishment have *a)* ignored the well-known facts related to the masking of the serum test for folate deficiency in the presence of vitamin-B_{12} deficiency and hemolysis accompanying malaria? And *b)* fallen prey to the false concept that folate deficiency is rare in resource-limited countries where dietary intake of folate and vitamin-B_{12} is poor, and anemia and malaria is endemic? Here we must confront our collective cognitive biases that have likely led to perpetuation of the medical myth exposed in this article.

Whenever a high-profile paper in the literature downplays key results from earlier papers, subsequent papers tend to highlight the more recent paper and exhibit "*recency bias*", wherein a disproportionate importance is given to more recent observations and conclusions.[87] This has, and will in turn, lead to an "*availability bias/cascade*"—a self-reinforcing process[87] wherein the collective beliefs of researchers appears more and more plausible through repetition of the same fact over and over again in high-profile journals. [Even Kipling's *Bandar-log* had a similar

slogan: "We all say so, so it must be true"[88]]. Intrinsic to the process of dissemination of medical information is the "*framing effect*", which (in the context of this paper) involves drawing different conclusions from the same information in a dataset, depending on the author's choice of presentation.[87] So when a normal-to-increased serum folate level is observed in vitamin-B_{12} deficiency, the potential of masked folate deficiency has been ignored.[36,46] Likewise the findings in Gambian and Malawian children with malaria and normal-to-high serum folate levels have subsequently led to broader conclusions that *almost all* African children with malaria do not have folate deficiency.[89] Then, as more and more experts from several diverse specialties—including obstetrics,[90] nutrition,[36] infectious disease,[91] internal medicine,[92] pediatrics,[69,93] cardiology,[94] endocrinology,[95] epidemiology and public health,[92] and <u>*even*</u> hematology[72,73]—studied populations at risk for both vitamin-B_{12} deficiency and malaria without sufficient consideration of all clinically relevant data including the dietary history, they referenced those papers that supported their own observations, and viewed the results from these cohorts through the narrow lens of their individual specialty. This reflects a "*confirmation bias"*— the tendency to search for or interpret the information of their own research in a way that confirms earlier preconceptions.[87] When confirmation bias reaches epidemic proportions,[87] this can lead to *in-group bias;* here reviewers of papers proffered to Journals can tend to preferentially accept those papers that reinforce the conclusions by several other members of their own group. Not surprisingly, further propagation of the concept among groups of researchers (now reading similar journals) will invariably lead to a "*bandwagon effect*" where many more individuals believe the same thing[87]—this is closely related to *groupthink* and herd behavior.[87] The danger of groupthink is particularly germane to various reviews or even consultations periodically held by major agencies (e.g., National Institutes of Health, World Health Organization, & Etc.) to generate "White Papers" on state-of-the-art. Minimization of controversy or conflict within this construct can fail to critically

assess the data that led to the conclusions in the first place, with a group-wise failure in accepting any alternative proffered ideas, views, or concepts.

Defining tissue folate deficiency in resource-limited countries — *caveats and limitations.* Because the liver parenchyma is the primary site for storage of folates[1], depletion of the amount of folate in the liver indicates folate deficiency; however a liver biopsy is much too invasive a test for routine clinical use. Given the limitations in interpretation of serum folate tests in resource-limited settings (as discussed above), can a study of other clinically accessible tissues help to confirm deficiency and perhaps distinguish it from vitamin-B_{12} deficiency? Although the test for RBC folates (by microbiological assay) was a good surrogate test for pure tissue folate stores, the newer non-microbiological assays for RBC folates have not been as clinically reliable (as discussed above). Moreover, in the context of a patient infected with malaria there are additional problems in interpretation of RBC folates. This stems from the fact that the malarial parasite has a life cycle that involves both an erythrocytic and an exo-erythrocytic phase (in liver) that results in hemolysis of red cells and lysis of infected parenchymal liver cells, respectively. And parasites themselves can contribute an [incompletely characterized] amount of folate to infected red cells.[59,60] This further limits the value of [serum and] RBC folates. So can an analysis of the bone marrow morphology [following a bone marrow aspirate/biopsy] identify megaloblastic anemia associated with folate deficiency? Yes, provided there are no other confounding variables. But in the case of associated malaria, information on the extent to which parasitic infestation *per se* modulates the morphological expression of folate deficiency in these tissues is limited to published data [on the morphology of bone marrow aspirates] from only a handful of children in The Gambia.[66,67] Because such studies were carried out during famine conditions (the Sahel Dessication), these children were likely to have had varying grades of multiple combined deficiencies of minerals (predominantly iron, but also zinc, copper, and

selenium) and micronutrients (including folate and vitamin-B_{12}, -A, -B_1, and -B_6). (Parenthetically the likelihood of multiple deficiencies in these children was not commented on in these publications).[66,67] Collectively, these multiple deficiencies can be expected to significantly alter the morphology of folate-deficient megaloblastic anemia. As one example, we know that associated iron deficiency will significantly modify and mask the [expected] classic features of megaloblastic anemia (i.e., masked megaloblastosis)[1]; instead, finding other subtle manifestations of giant myelocytes and metamyelocyte and hypersegmented polymorphonuclear leukocytes are clues that can clinch the diagnosis. [Therefore preclinical studies that can precisely clarify the independent and combined impact of additional mineral and micronutrient deficiencies in modifying the expected features of megaloblastic anemia, and how this is further altered in malaria-infected bone marrow, are areas for additional study]. Finally, merely identifying megaloblastic anemia cannot distinguish between folate or vitamin-B_{12} deficiency.[1] Thus, the challenge to unambiguously diagnose single deficiency of folate or vitamin-B_{12} from combined vitamin-B_{12} and folate deficiency necessarily rests on the cumbersome and expensive serial measurement of metabolites (methylmalonic acid and homocysteine) after specific replacement of the deficient vitamin (as discussed above). But by the same token, mere use of a single metabolite test result to exclude the coexistence of folate deficiency warrants far greater caution in interpretation than exhibited in the publications cited.

Alternative methods for assessing folate status in resource-limited settings. Dietary assessment using a variety of methods (24-h recall, estimated/weighed record, or locally validated food-frequency questionnaires[96-98]) are established methods to evaluate the quality of food and quantity of nutrients consumed by individuals in an ethnically homogeneous area.[4,99] Despite intrinsic limitations, these methods can predict with fair reliability the dietary folate and vitamin-B_{12} intake of a larger population consuming similar foods, and identify those at risk for

nutritional insufficiency.[4,45,48,96-98,100] These approaches allow for the conclusion that there is inadequate dietary-folate intake and widespread folate deficiency throughout India, which is also associated with both iron and vitamin-B_{12} deficiency.[35,45,48,96,101] Such information contributed to raising the recommended dietary allowance for folate intake in India to 250-μg/day in 2010.[102] Similar approaches can be used in other resource-limited countries.

On the consequences of underestimating poor folate status in women and children. Anemia is the most common clinical manifestation of a deficiency of iron-, folate-, and vitamin-B_{12} (in various combinations); is widespread among children, adolescents, and women throughout India[103,104]; is believed to be among the highest in the world[101,105,106]; and can lead to serious maternal morbidity and mortality.[49,107-110] Likewise, the relatively monotonous diet of women and children and the accompanying food insecurity in Africa—in Kenya,[97,100,111,112] Malawi,[77,78,80] and The Gambia[68] that predisposes them to clinically significant deficiencies of iron, folate and vitamin-B_{12}[7,37,44,63]—also manifests as widespread nutritional anemia.[103] In this context, it should be noted that attempts to replenish either iron and/or vitamin-B_{12} without addressing existing folate deficiency would invariably result in persistent anemia.

Although there are many causes for neural-tube defects, the vast majority of these defects can be prevented by periconceptional folate supplements[1,6,113-115] with greater protection (up to 70%) afforded when the incidence of neural-tube defects is higher.[114] Based on both preclinical studies[116-119] and clinical trials[113,114], the relationship between folate replacement and prevention of neural-tube defects fulfills Koch's postulates on the causation of disease.[120] Therefore the value of optimizing folate status is self-evident. But is this a problem in India? The only population-based approach reported from a cluster of villages in North India discovered the incidence of neural-tube defects was 6·57- to 8·21- per 1000-live births,[121] which is among the highest in the world. Although our study was from the least-developed area of India[121], this region was

representative of 168 additional districts in India, and the incidence of neural-tube defects was similar to that recorded in tertiary centers from several major cities in North and South India[122-124]; this somewhat validates extrapolation of the incidence of neural-tube defects to a much wider area in India.[121] A recent systematic review has identified a birth prevalence of neural-tube defects in India of 4·1 per 1000-births,[125] reflecting a largely silent epidemic of mammoth proportions.[121,126] Therefore, using data from the lower and higher incidence of neural-tube defects (4·1- to 8·21- per 1000-births), and conservative estimates on potential prevention of one-half of neural-tube defects with periconceptional folate supplements[114]—based on an estimated 27-million annual pregnancies in India—there could well be between 55,000- to 110,000-births with neural-tube defects that can be prevented *each year* by optimizing folate status. This is a critically important opportunity for governmental leaders to rectify[2] and thereby reduce the incalculable physical, psychological and economic burden for affected children and their families.

(Despite the strength of the data underlying the prevention of neural-tube defects with folate supplementation, because of the close metabolic interrelationship between folate and vitamin-B_{12}, it is possible—(but not yet unambiguously proven clinically)—that vitamin-B_{12} deficiency will also be independently shown as a cause of neural tube defects.[127] Clinical investigations into the potential role of vitamin-B_{12} deficiency as an independent risk factor in neural-tube defects—based on low vitamin-B_{12} levels in mothers of affected progeny—will necessarily need to rule out an associated [masked] folate deficiency. So despite the apparent high prevalence of vitamin-B_{12} deficiency, it is not yet possible to attribute a precise risk percentage of vitamin-B_{12} deficiency to neural-tube defects in India).

Prophylactic administration of folate to women of childbearing age can prevent short-term *pregnancy complications* (anemia, abruptio placenta, small-for-date babies, preterm birth),

as well as adverse short-term *pregnancy outcomes* for the newborn (low birthweight, midline birth defects).[128] Recent studies from UK, Norway, Sweden, and India—where government-mandated folate fortification of food is not in practice—also suggest that the progeny of women who do not consume folate supplements during the periconceptional period can exhibit subtle long-term adverse consequences in behavior during childhood.[129-132] Parenthetically, these observations are the human correlates of earlier preclinical studies (in mice),[133,134] which confirmed a relationship between gestational folate deficiency and abnormal neuro-pathology and neuro-development *in utero*. Our subsequent studies suggest a novel molecular basis for the aberrant activation of such neurological pathways in the fetal brain that experiences gestational folate deficiency; this involves the post-translational homocysteinylation of an mRNA-binding protein that abnormally modulates the expression of proteins that are involved in the biosynthesis of both neurotransmitters and neurons.[1,6,135] This is yet another area that warrants additional study.

Appropriate prophylaxis negates laboratory testing for folate deficiency. The mandate for folate supplementation of patients with hemolysis originally arose in the West, where despite consumption of an apparently folate-sufficient diet, patients with different etiologies for hemolysis often developed folate deficiency that led to an aplastic crisis.[8,13] This hemolysis-induced folate deficiency predictably arises from increased requirements to support compensatory hematopoiesis in the bone marrow and chronic loss of red cell folates into the urine and stool. Therefore a longstanding dictum in clinical hematology is that <u>*all*</u> patients with hemolysis from any cause must be given 1-mg folic acid/day to optimize hematopoiesis, because a failure to do so can precipitate a reticulocytopenic [megaloblastic] crisis.[1,6] [Additional causes for hemolysis in patients with malaria that warrant routine folate supplements include: hemoglobinopathies that confer resistance to malaria (α- or β-thalassemia, sickle cell disease);

individuals with glucose 6-phosphate dehydrogenase-deficiency given primaquine, sulfadoxine, or dapsone for malaria; a Coombs-positive immune-mediated hemolysis; and sepsis-induced disseminated intravascular coagulation]. Parenthetically, and in this context, among populations where the dietary intake of folate, vitamin-B_{12}, and iron is compromised, it is unknown how frequently the reticulocytopenia observed in *Plasmodium falciparum*-anemia[74] can be reversed with folate *plus* vitamin-B_{12} *plus* iron supplements. As noted above, it is possible that other associated micronutrients and mineral deficiencies can profoundly alter hematopoiesis in the bone marrow. Nevertheless, there has been *no* controlled clinical trial that has unambiguously shown that such children did not benefit from replacement doses of folic acid administered with antimalarials after iron and/or vitamin-B_{12} deficiency is reversed. Therefore until such data is available, children suspected of having such micronutrient deficiencies ought to be given the benefit of such replacement.

A related axiom in clinical medicine is that there is little point in performing any test if the result does not change one's therapeutic decision. Therefore a relevant question is: "Why is it necessary to know the folate status of an individual with active malaria, since all such patients *ought* to be given folate to support compensatory hematopoiesis (together with antimalarials)?" Indeed, one can make a strong case that in clinical practice in resource-limited settings the use of blood tests to assess for folate status during hemolysis has no basis.

But are there risks to administering folate supplements to support compensatory hematopoiesis in malaria? A recent meta-analysis of 19 studies have put to rest the concern of iron-folate and malarial progression[136] and a Cochrane analysis identified *no* evidence that this is a problem when combined with antimalarial drugs and insecticide-treated bed-nets.[137]

Although *Plasmodium falciparum* possess two folate transporter proteins that can facilitate membrane transport of folic acid, folinic acid, the folate precursor p-amino benzoic

acid (pABA), and the human folate catabolite $pABAG_n$, rescue experiments on parasites *in vitro* show that pABA was the only effective salvage substrate at normal physiological levels.[138] Recently Van Eijk *et al*[139] affirmed the safety of low-dose folic acid (1-mg/day) in 467-pregnant women with malaria; however, high-dose folic acid (5-mg/day) will allow for resistance, and have adverse outcomes in children.[140-142]

Therefore given their propensity to have multiple deficiencies that continue into pregnancy, all non-pregnant women who require anti-malarial treatment[143] should be treated for both malaria as well as iron, folate, and vitamin-B_{12} to enable optimal hematopoiesis. Likewise, all pregnant women living in resource-limited malarious areas malaria should routinely be given intermittent preventive treatment according to the latest guidelines (currently it is sulfadoxine-pyrimethamine[144]) together with insecticide-treated bednets, plus 1-mg folic acid, prophylactic doses of 10- to 25-μg vitamin-B_{12} and replacement doses of iron orally.[145]

Finally, optimizing the nutrition and general health of young women well before they are pregnant (i.e., intentional preparation for pregnancy[146]) is also the best way to ensure optimum transfer of nutrients to the developing fetus, and to nurture the baby after delivery. Indeed, this is the only way to curtail the inter-generational passage of these mineral and micronutrient deficiencies from mother to baby *ad infinitum*.

Conclusion

The repetition of a critical error involving the misinterpretation of a blood test result in the clinical literature in populations at risk for combined folate- and vitamin-B_{12}- deficiency and/or malaria has led to a 'snowball effect' wherein paper-upon-paper has propagated the myth that folate deficiency is uncommon in resource-limited settings. As a result, millions of women and children with true [tissue] folate deficiency have probably not been given folate replacement. It is hoped that recognition and redress of such serious errors among women and children in these

resource-limited settings—that can be achieved by early prophylactic replenishment with micronutrients that include folate and vitamin-B_{12} (and key minerals)—will improve maternal-child health and reduce the unacceptably high morbidity and mortality in these regions.

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***Legend to Figure* 1: Vitamin-B_{12} and folate interrelationships:** Normally, the tissue utilization of serum 5-methyl-tetrahydrofolate requires vitamin-B_{12} (as methyl-vitamin-B_{12} [also known as methyl-cobalamin]) to activate methionine synthase, a key enzyme that converts 5-methyl-tetrahydrofolate to tetrahydrofolate, which is then polyglutamylated by folylpolyglutamate synthase (not shown in the figure). These folate polyglutamates are thereby retained in the cell and are the forms that participate in and perpetuate one-carbon metabolism. But with vitamin-B_{12} deficiency, there is lowered activity of methionine synthase, which results in a functional folate deficiency wherein an increased amount of 5-methyl-tetrahydrofolate—(and homocysteine that is not methylated by methionine synthase to form methionine)—is trapped and accumulates within cells. Because 5-methyl-tetrahydrofolate is a poor substrate for folylpolyglutamate synthase, there is decreased synthesis of folate polyglutamates. As a result 5-methyl-tetrahydrofolate (and homocysteine) leaks out of cells into blood.[1,21] When vitamin-B_{12} deficiency is prolonged, there is a real cellular folate deficiency arising from failure to retain folate within cells.[21] This explains why patients with pernicious anemia and/or nutritional vitamin-B_{12} deficiency have *normal to high* serum folates (that can mask an associated mild-to-moderate folate deficiency) and high serum homocysteine levels, and an initial functional intracellular folate deficiency that culminates over time in a true tissue folate deficiency, and low erythrocyte folates.

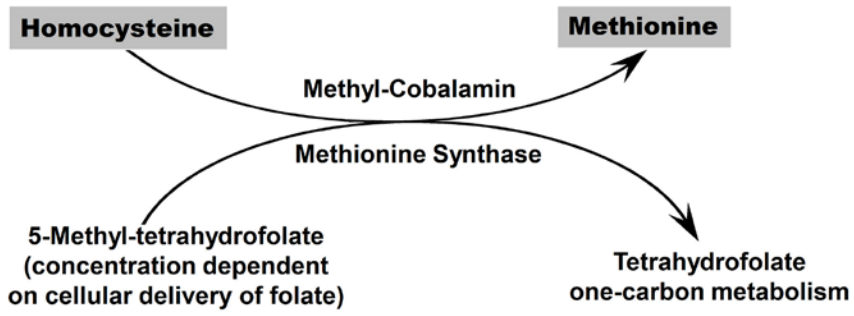

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Table* 1. Serum folates are Misleadingly Elevated in Vitamin-B_{12} Deficiency and/or Malaria Which Are Both Common in Resource-Limited Settings§ (From Ref. [6])

	Serum folates	***Erythrocyte folates***	***Serum Vitamin-B_{12}***
Pure Folate deficiency	Low	Low	Normal/Low*
Pure Vitamin-B_{12} deficiency$^{\Psi}$	Normal/High*$^{\Psi}$	Low*$^{\Psi}$	Low
Folate *plus* Vitamin-B_{12} deficiency	Normal*	Low	Low
Pure Malaria	Normal*/High*$^{\xi}$	High*$^{\Phi\zeta}$	Normal
Malaria *plus* Folate deficiency	Normal*	Normal*/High*/Low	Normal
Malaria *plus* Vitamin-B_{12} deficiency	Normal*/High*$^{\xi}$	Normal*/High*$^{\zeta}$	Low
Malaria *plus* Folate *plus* Vitamin-B_{12} deficiency	Normal*/High*$^{\xi}$	Low/Normal*$^{\zeta\,\Omega}$	Low

* The *asterisk* indicates misleading values in the clinical settings shown on the *left*.

§ Both vitamin-B_{12} deficiency and clinical malaria and other hemolytic states can complicate the diagnosis of folate deficiency using tests for serum- or erythrocyte- folate concentration.[147]

$^{\Psi}$ Vitamin-B_{12} deficiency is accompanied by inability to utilize folates for one-carbon metabolism so folates leak out of erythroid precursors into serum.

$^{\xi}$ Release of the 30-fold excess folate from infected erythroid precursors, reticulocytes, and mature erythrocytes during hemolysis raises serum folate levels. (An as-yet-unknown quantity of folate is released into serum when folate-rich hepatocytes are destroyed during the exo-erythrocytic hepatic phase of malaria).

$^{\Phi}$ Hemolysis induces a compensatory reticulocytosis; these reticulocytes are richer in folate than mature erythrocytes.[14,56,58]

$^{\zeta}$ *Plasmodium falciparum* can also synthesize folates in erythrocyte cultures *in vitro*[59] and raises erythrocyte folates in animal models with high levels of parasitemia.[60]

$^{\Omega}$ Reticulocytopenia in severe *Plasmodium falciparum* malaria, due to either combined vitamin-B_{12} deficiency *plus* folate deficiency, which can trigger a reticulocytopenic (megaloblastic) crisis], or cytokine-induced inhibition of hematopoiesis, will negate an expected rise in erythrocyte folates.

***Table* 2.**
Interpretation of test results on serum methylmalonic acid and total homocysteine
(adapted from Ref. [1])

Methylmalonic Acid*	**Total Homocysteine**[§ ξ]	**Diagnosis**
Increased	Increased	Vitamin-B_{12} deficiency confirmed; folate deficiency *still* possible (i.e., combined vitamin-B_{12} *plus* folate deficiency is possible)[ξ]
Normal	Increased	Folate deficiency is likely
Normal	Normal	Vitamin-B_{12} and folate deficiency is excluded

* Methylmalonic acid (normal values = 70–270 nM)

§ Homocysteine (normal values = 5–14 μM)

ξ In patients with combined folate *plus* vitamin-B_{12} deficiency, the serum homocysteine will not fall completely into the normal range unless both folate and vitamin-B_{12} are replaced.[34] Less commonly, with pyridoxine deficiency, specific replacement with pyridoxine will be required before the serum homocysteine falls into the normal range.